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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

AEM96-01A

U.S. APPLICATION NO. (if known, see 37 C.F.R.1.5)

INTERNATIONAL APPLICATION NO.

PCT/IL96/00143

INTERNATIONAL FILING DATE

06 November 1996 (06-11-96)

PRIORITY DATE CLAIMED

06 November 1995

TITLE OF INVENTION

In Vitro Construction of SV40 Viruses and Pseudoviruses

APPLICANT(S) FOR DO/EO/US

Ziv Sandalon, Amos B. Oppenheim, Ariella Oppenheim

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
 2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
 3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
 4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
 6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
 7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
 8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
 9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (executed, 3 pgs)
 10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).
- Items 11. to 16. below concern document(s) or information included:
11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
 12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
 13. ☒ A **FIRST** preliminary amendment with replacement pages
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
 14. ☐ A substitute specification.
 15. ☐ A change of power of attorney and/or address letter.
 16. ☐ Other items or information:

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) PCT/IL96/00143	INTERNATIONAL APPLICATION NO. 06 November 1996 (06-11-96)	ATTORNEY'S DOCKET NUMBER AEM96-01A
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17. ☒ The following fees are submitted after entry of Pre. Amendment:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO..... \$ 930.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)..... \$ 720.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).. \$ 790.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$ 1070.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... \$ 98.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	46 -20 =	26	x 22.00	\$	572 00
Independent claims	4 -3 =	1	x 82.00	\$	82 00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				\$	270 00
TOTAL OF ABOVE CALCULATIONS =				\$	1724 00
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).					
SUBTOTAL =				\$	1724 00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE =				\$	1724 00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +					
TOTAL FEES ENCLOSED =				\$	1724 00
				Amount to be:	
				refunded	\$
				charged	\$

CALCULATIONS **PTO USE ONLY**

a. ☒ A check in the amount of \$ 1724.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-0380. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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REGISTRATION NUMBER

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09/068293

PATENT APPLICATION
Docket No.: AEM96-01A

IN THE UNITED STATES RECEIVING OFFICE (RO/US)

Designated/Elected Office (DO/EO/US)

U.S. National Phase of

International Application No.: PCT/IL96/00143

International Filing Date: November 6, 1996

Priority Date Claimed: November 6, 1995

Applicants: Ziv Sandalon, Amos B. Oppenheim, Ariella Oppenheim

Title: *In Vitro* Construction of SV40 Viruses and Pseudoviruses

Attorney's Docket No.: AEM96-01A

Express Mail: EM080920391US

Dated: 6 MAY 1998

PRELIMINARY AMENDMENT

In the Specification:

On page 1, please insert the following Related Applications paragraph after the Title and before the Field of Invention:

---RELATED APPLICATION(S)

This application is the U.S. National Phase of PCT/IL96/00143, filed on November 6, 1996; which claims priority to Israeli Application No.: 115880 filed on November 6, 1995, the entire teachings of which are incorporated herein by reference.---

In the Claims:

Please cancel Claims 1-46 found on pages 35-42 of PCT/IL96/00143 and insert Claims 1-46 attached herewith as substitute pages 35-45.

REMARKS

The Related Applications paragraph is being added to indicate that the instant Application is the U.S. National Phase of International Application PCT/IL96/00143 filed on November 6, 1996, which claims priority to Israeli Application No.: 115880 filed on November 6, 1995.

Claims 1-46, found on pages 35-42 of PCT/IL96/00143 are being replaced with Claims 1-46 attached herewith as substitute pages 35-45 to conform with U.S. Patent practice.

No new matter has been added.

Respectfully submitted,

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Dated: May 6, 1998
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-35-

CLAIMS

What is claimed is:

1. A construct capable of infecting a mammalian cell comprising at least one semi-purified or pure SV40 capsid protein; and a constituent selected from the group consisting of:
- a) an exogenous DNA encoding a therapeutic exogenous protein or peptide product, or encoding therapeutic RNA, or itself a therapeutic product,
 - 10 b) a vector comprising an exogenous DNA encoding a therapeutic exogenous protein or peptide product, or encoding therapeutic RNA, or itself a therapeutic product,
 - c) an exogenous RNA encoding a therapeutic exogenous protein or peptide product or itself a therapeutic product,
 - 15 d) vector comprising an exogenous RNA encoding a therapeutic exogenous protein or peptide product or itself a therapeutic product,
 - e) therapeutic exogenous protein or peptide product, and
 - 20 f) antisense RNA, ribozyme RNA or any RNA or DNA which inhibits or prevents the expression of undesired protein or proteins in said mammalian cell; and further comprising operatively linked
 - 25 regulatory elements sufficient for the expression

-36-

and/or replication of said exogenous protein in a mammalian cell.

2. A construct according to Claim 1 further comprising additional SV40 protein or proteins, preferably SV40 agnoprotein.
5
3. A construct according to Claim 1 comprising a mixture of at least two semi-purified or pure SV40 capsid proteins.
4. A construct according to Claim 1 comprising a mixture of three semi-purified or pure SV40 capsid proteins.
10
5. A construct according to Claim 1 wherein said SV40 capsid protein is semi-purified or pure VP1 or VP2 or VP3.
6. A construct according to Claim 1 wherein said constituent is exogenous circular or linear DNA encoding a therapeutic exogenous protein or peptide product, or itself a therapeutic product, or encoding therapeutic RNA, or a vector comprising exogenous DNA encoding therapeutic RNA or encoding a therapeutic exogenous protein or peptide product.
15
20
7. A construct according to Claim 6 wherein said DNA is DNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is DNA
25

-37-

which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount, or encodes a therapeutic RNA.

- 5 8. A construct according to Claim 7 wherein said therapeutic protein or peptide product is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.
- 10 9. A construct according to Claim 1 comprising SV40-derived ori DNA sequence as a replication regulatory element and further comprising a DNA sequence encoding one or more regulatory elements sufficient for the expression of said exogenous RNA or exogenous protein or peptide in said mammalian cell.
- 15 10. A construct according to Claim 1 wherein said constituent is exogenous RNA, wherein said RNA is RNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is RNA which encodes a therapeutic protein or peptide product
- 20 which is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein or peptide product which is made contained in said cell in defective form, or is RNA which encodes a therapeutic protein or peptide product which is made
- 25 or contained in said cell in physiologically abnormal or normal amount, said RNA having regulatory elements, including translation signal or signals sufficient for the translation of said protein or peptide product in said mammalian cell, operatively linked thereto.

11. A construct according to Claim 10 wherein said therapeutic protein or peptide product is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.
- 5 12. A construct according to Claim 1 wherein said constituent is a therapeutic exogenous protein or peptide product which is, respectively, a therapeutic protein or peptide product which is not made or contained in said cell, or is a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is a therapeutic protein or peptide product which is made or contained in said cell in defective form or is a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount.
- 10
13. A construct according to Claim 1 wherein said constituent is antisense RNA or DNA or ribozyme RNA. or any RNA or DNA which inhibits or prevents the expression of undesired protein or proteins in said mammalian cell.
- 15
- 20
14. A construct according to Claim 13 wherein said antisense RNA is antisense RNA directed against the *bcr/abl* transcript.
- 25 15. A construct according to Claim 13 wherein said antisense RNA is antisense RNA directed against a HIV transcript.
16. A construct according to Claim 1 wherein said cell is a human cell selected from the group consisting of

-39-

hemopoietic cells, epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells. tumor cells, nerve cells and germ line cells.

17. A construct according to Claim 16 wherein said
5 hemopoietic cells are bone marrow cells, peripheral blood cells and cord blood cells, or liver cells.
18. A method for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid comprising the following steps:
- 10 a. allowing a semi-purified or pure SV40 capsid protein or a mixture of at least two such proteins to self-assemble into SV40-like particles; and
- 15 b. bringing the SV40-like particles assembled in step (a) into contact with said exogenous nucleic acid to give recombinant SV40 viruses or with a vector comprising said exogenous nucleic acid to give pseudoviruses.
19. The method of Claim 18 wherein said recombinant SV40
20 viruses or pseudoviruses are subjected to digestion by nuclease to remove non-packaged DNA.
20. A method according to Claim 18 wherein in step (a) at
25 least one other SV40 protein, preferably SV40 agnoprotein, is added to the mixture of said SV40 capsid protein or proteins and said nucleic acid.

21. A method according to Claim 18 wherein said SV40 capsid protein is semi-purified or pure SV40 VP1, VP2, or VP3.
22. A method according to Claim 18 wherein said exogenous nucleic acid is circular or linear DNA.
23. A method according to Claim 18 wherein said exogenous nucleic acid is RNA.
24. A method according to Claim 18 wherein said exogenous nucleic acid encodes a therapeutic protein or peptide product or itself a therapeutic product.
25. A method according to Claim 22 wherein said DNA is DNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount or is DNA which encodes a therapeutic RNA.
26. A method according to Claim 25 wherein said exogenous DNA encodes a therapeutic protein or peptide product which is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.
27. A method according to Claim 18 wherein in step (b) SV40-derived *ori* DNA sequence is added and said

-41-

exogenous nucleic acid has DNA sequence encoding one or more regulatory elements sufficient for the expression of said exogenous protein in said mammalian cell operatively linked thereto.

- 5 28. A method according to Claim 18 wherein said nucleic acid is exogenous RNA, wherein said RNA is RNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is RNA which encodes a therapeutic protein or peptide product which
10 is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is RNA which encodes a therapeutic protein or peptide product which is made
15 or contained in said cell in physiologically abnormal or normal amount and wherein said RNA has regulatory elements, including translation signal, sufficient for the translation of said protein product in said mammalian cell, operatively linked thereto.
- 20 29. A method for the *in vitro* construction of recombinant SV40 viruses or pseudoviruses comprising an exogenous therapeutic protein or peptide comprising the following steps:
- 25 a. allowing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins to self-assemble into SV40-like particles; and
- b. bringing the SV40-like particles assembled in step (a) into contact with said exogenous protein

-42-

to give recombinant SV40 viruses or
pseudoviruses.

30. A method according to Claim 29 wherein said
recombinant SV40 viruses or pseudoviruses are purified
5 from any non-packaged protein.

31. A method according to Claim 29 wherein said exogenous
protein or peptide are, respectively, a naturally
occurring or recombinant protein or peptide, a
chemically modified protein or peptide, or a synthetic
10 protein or peptide.

32. A method according to Claim 31 wherein said exogenous
protein or peptide product is, respectively, a
therapeutic protein or peptide product not made or
contained in said cell, or are a therapeutic protein
or peptide product made or contained in said cell in
15 abnormally low amount, or are a therapeutic protein or
peptide product made or contained in said cell in
defective form or are a therapeutic protein or peptide
product made or contained in said cell in
20 physiologically abnormal or normal amount.

33. A method according to Claim 32 wherein said cell is a
human cell selected from the group consisting of
hemopoietic, cells, muscle cells, tumor cells, nerve
cells and germ line cells.

25 34. A method according to Claim 33 wherein said
hemopoietic cells are bone marrow cells, peripheral
blood cells and cord blood cells, or liver cells.

35. A method for the *in vitro* construction of SV40 pseudoviruses comprising exogenous antisense RNA, or ribozyme RNA or RNA or DNA which inhibits or prevents the expression of undesired protein or proteins in a mammalian cell, comprising the following steps:
- 5
- a. allowing a semi-purified or pure SV40 capsid protein or a mixture of at least two such proteins to self assemble into SV40-like particles and
- 10 b. bringing said SV40-like particles obtained, in step (a) into contact with said exogenous antisense RNA, or ribozyme RNA, or RNA or DNA which inhibits or prevents the expression of undesired protein/s in a mammalian cell, to give
- 15 recombinant SV40 pseudoviruses.
36. The method of Claim 35 wherein said pseudoviruses are subjected to digestion by nuclease to remove non-packaged DNA.
- 20 37. A method according to Claim 35 wherein in step (a) at least one other SV40 protein, preferably SV40 agnoprotein, is added to the mixture of SV40 capsid protein or proteins and the exogenous nucleic acid or antisense nucleic acid.
- 25 38. A method according to Claim 35 wherein said SV40 capsid protein is semi-purified or pure SV40 VP1, VP2, or VP3.

-44-

39. A method according to Claim 35 wherein said antisense RNA is antisense RNA directed against the *bcr/abl* transcript.
- 5 40. A method according to Claim 35 wherein said antisense RNA is antisense RNA directed against a HIV transcript.
41. A mammalian cell infected with a construct of Claim 1.
- 10 42. An infected human cell according to Claim 41 selected from the group consisting of hemapoietic cells, muscle cells, tumor cells, nerve cells and germ line cells.
- 15 43. A method of providing a therapeutic DNA, RNA, antisense RNA, ribozyme RNA, protein or peptide product to a patient in need of such product by administering to said patient a therapeutically effective amount of the SV40 viruses or pseudoviruses according to Claim 1.
- 20 44. A method of providing a therapeutic, DNA, RNA, antisense RNA. ribozyme RNA, protein or peptide product to a patient in need of such product by administering to said patient a therapeutically effective amount of infected cells according to Claim 41.
- 25 45. Pharmaceutical compositions comprising as active ingredient a therapeutically effective amount of the SV40 viruses or pseudoviruses according to Claim 1.

IN-VITRO CONSTRUCTION OF SV40 VIRUSES AND
PSEUDOVIRUSES

FIELD OF THE INVENTION

- 5 The invention relates to methods of *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid or exogenous protein or peptide which are particularly suitable for use in gene therapy.

BACKGROUND OF THE INVENTION

- 10 Previous studies have shown that SV40 virions disrupted at pH 10.6 [Christensen, M. & Rachmeler, M. (1976) *Virology* 75:433-41] or by reducing disulfide bonds [Colomar, M.C., *et al.* (1993) *J. Virol.* 67:2779-2788] may be reassociated to form infectious SV40 aggregates. The early attempts to package *in vitro* foreign DNA in these aggregates [Christensen & Rachmeler (1976) *ibid.*] produced infectious
15 products which did not resemble SV40 virions. Furthermore, their resistance to DNase has not been tested. Later, *in vitro* packaging experiments [Colomar *et al.* (1993) *ibid.*] did not yield particles with infectivity above the level of naked DNA.

- Recently, pseudocapsids of the closely related murine polyoma virus, prepared from polyoma VP1, were used as carriers for heterologous DNA into mammalian cells
20 [Forstova, J., *et al.* (1995) *Hum. Gene Therapy* 6:297-306]. The pseudo-capsid protected 2-30% of the input DNA from DNase I digestion. When a plasmid carrying the *cat* gene was tested, most of the DNA which was protected from DNase I appeared as a ~2kb fragment, while the input plasmid was significantly
25 partially protected against DNases. Infectious units were not measured in those

experiments. The DNA transferred into recipient cells was functional in gene expression, albeit at a very low efficiency. With a 1.6kb DNA fragment which carries the polyoma middle T-antigen, <30 transformed foci were obtained per 1µg of input DNA. Similarly, a low level of CAT activity was observed with the plasmid carrying the *cat* gene.

SV40 is a simian papovirus, with a small double-stranded circular DNA genome of 5.2kb [reviewed in Tooze, J. (1981) DNA Tumor Viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York]. The viral capsid, surrounding the viral mini-chromosome, is composed of three viral-coded proteins, VP1, VP2, and VP3. Recent X-ray crystallographic studies on SV40 structure at 3.8Å resolution [Liddington, R., *et al.* (1991) Nature **354**:278-282] revealed that the outer shell of the virion particle is composed of 72 pentamers of VP1, 60 hexavalent and 12 pentavalent. The VP2 and VP3 appear to bridge between the VP1 outer shell and the chromatin core. The VP1 pentamers have identical conformations, except for the carboxy-terminal arms, which tie them together. Five arms extend from each pentamer and insert into the neighboring pentamers in three distinct kinds of interactions. It appears that this construction facilitates the use of identical building blocks in the formation of a structure that is sufficiently flexible as required for the variability in packing geometry [Liddington *et al.* (1991) *ibid.*].

Another protein encoded by the late regions of SV40 (which also encoded the three capsid proteins VP1, VP2 and VP3) is the agnoprotein, also called LP1. This is protein a small, 61 amino acid protein. Although the agnoprotein was not found in the viral capsid, it is thought to expedite viral assembly *in vivo* [Resnick, J & Shenk, T. (1986) J. Virol. **60**:1098-1106; Ng, S.C., *et al.* (1985) J. Biol. Chem. **260**:1127-1132; Carswell, S.& Alwine, J. C. (1986) J. Virol. **60**:1055-1061].

The major hindrance in beginning to use the SV40 pseudovirions in preliminary experiments in humans is the present need for a viral helper for encapsidation. This results in pseudoviral stocks that contain also wild type SV40. Because of the

similarity in properties (shape, size and density) between the pseudovirions and the helper, they cannot be separated by physical means. An ideal way to prepare pseudovirions for therapeutic purposes for human use would be by *in vitro* packaging. This would provide maximal safety, since all steps of the preparation can be well controlled. *Ex vivo* administration would circumvent problems associated with immune response.

Viral packaging *in vivo* occurs by gradual addition and organization of capsid proteins around the SV40 chromatin [Garber, E.A., *et al.* (1980) *Virology* **107**: 389-401; Bina, M. (1986) *Comments Mol. Cell Biophys.* **4**:55]. The three capsid proteins VP1, VP2 and VP3 bind to DNA non-specifically [Soussi, T. (1986) *J. Virol.* **59**:740-742; Clever, J., *et al.* (1993) *J. Biol. Chem.* **268**:20877-20883]. How the specific recognition between the viral capsid proteins and its DNA is achieved remains unclear. The packaging of SV40 using pseudovirions, in which most of the viral DNA is replaced by other sequences has been investigated [Oppenheim, A., *et al.* (1986) *Proc. Natl. Acad. Sci. USA* **83**:6925-6929]. The pseudoviral particles are prepared by encapsidating plasmids that carry the SV40 origin of replication (*ori*) and the packaging signal (*ses*) [Oppenheim, A., *et al.* (1992) *J. Virol.* **66**:5320-5328]. The model suggests that *ses* serves several functions in SV40 packaging: as a sensor for the level of the late viral proteins in the transition from replication and/or transcription to packaging, in nucleosomal reorganization and the initiation of viral assembly [Oppenheim, A., *et al.* (1994). *J. Mol. Biol.* **238**:501-513] and probably also as a nucleation center for viral assembly [Dalyot-Herman, N. *et al.* (1996) *J. Mol. Biol.* **259**:69-80].

The pseudovirions, carrying various genes of therapeutic interest, are very efficient in DNA transfer into a wide range of cells, including human bone marrow cells, and are therefore potential vectors for gene therapy [Oppenheim *et al.* (1986) *ibid.*; Oppenheim A., *et al.* (1987) *Ann. New York Acad. Sci.* **511**:418-427; Dalyot, N. & Oppenheim, A. (1989) Efficient transfer of the complete human beta-globin gene

into human and mouse hemopoietic cells via SV40 pseudovirions. In: Gene Transfer and Gene Therapy (Beaudet, A.L., Mulligan R., I.M. Verma, eds), pp. 47-56, Alan R. Liss, Inc., New York; Oppenheim, A., *et al.* (1992) Development of somatic gene therapy: A simian virus 40 pseudoviral vector for hemopoietic cells. In Genetic
5 Among Jews (Bonne-Tamir, B., A. Adams, eds), pp. 365-373, Oxford University Press, Oxford]. The ideal way to prepare pseudovirions for therapeutic purposes for human use is by *in vitro* packaging. This would provide maximal safety, since all steps of the preparation can be well controlled.

10 SUMMARY OF THE INVENTION

The present invention relates to construct capable of infecting a mammalian cell comprising at least one semi-purified or pure SV40 capsid protein and a constituent selected from the group consisting of an exogenous DNA encoding an exogenous protein or peptide product, or encoding therapeutic RNA, or itself a therapeutic
15 product, a vector comprising an exogenous DNA encoding an exogenous protein or peptide product, or encoding therapeutic RNA, or itself a therapeutic product, an exogenous RNA encoding an exogenous protein or peptide product or itself a therapeutic product, a vector comprising an exogenous RNA encoding an exogenous protein or peptide product or itself a therapeutic product, an exogenous
20 protein or peptide product, and antisense RNA, ribozyme RNA or any RNA or DNA which inhibits or prevents the expression of undesired protein/s in said mammalian cell; and optionally further comprising operatively linked regulatory elements sufficient for the expression and/or replication of said exogenous protein in a mammalian cell.

25 The construct of the invention may optionally further comprise additional SV40 protein or proteins, preferably SV40 agnoprotein.

Constructs according to the invention may comprise as said constituent exogenous circular or linear DNA encoding an exogenous protein or peptide product, or is itself a therapeutic product, or a vector comprising exogenous DNA encoding a therapeutic RNA, or encoding an exogenous protein or peptide product.

- 5 The said protein product is preferably a therapeutic protein or peptide product which is not made or contained in mammalian cells, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in such cells in abnormally low amount, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in such cells in defective form or is DNA which
- 10 encodes a therapeutic protein or peptide product which is made or contained in mammalian cells in physiologically abnormal or normal amount and can be an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

- The constructs of the invention may comprise SV40-derived *ori* DNA sequence as said replication regulatory element and may further comprise DNA sequences
- 15 encoding one or more regulatory elements sufficient for the expression of said exogenous RNA or exogenous protein or peptide in said mammalian cell.

- In other embodiments, in constructs according to the invention said constituent is exogenous RNA, preferably RNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is RNA which encodes a
- 20 therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein or peptide product which is made contained in said cell in defective form, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount, said RNA having regulatory
- 25 elements, including translation signal/s sufficient for the translation of said protein or peptide product in said mammalian cell, operatively linked thereto.

In other embodiments, the constructs according to the invention may comprise as said constituent an exogenous protein or peptide product, which can be a therapeutic protein or peptide product which is not made or contained in mammalian cells, or is a therapeutic protein or peptide product which is made or contained in such cells in abnormally low amount, or is a therapeutic protein or peptide product which is made or contained in such cells in defective form or is a therapeutic protein or peptide product which is made or contained in mammalian cells in physiologically abnormal or normal amount.

In further embodiments, the constructs according to the invention may comprise as said constituent antisense RNA or DNA or ribozyme RNA, or any RNA or DNA which inhibits or prevents the expression of undesired protein/s in mammalian cells.

The mammalian cells can be hemopoietic cells, such as bone marrow cells, peripheral blood cells and cord blood cells or liver cells., epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells, tumor cells, nerve cells and germ line cells.

The invention further relates to a method for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid comprising the steps of bringing a semi-purified or pure SV40 capsid protein or a mixture of at least two such proteins into contact with the exogenous nucleic acid to give recombinant SV40 viruses or with a vector comprising the exogenous nucleic acid to give pseudoviruses; and optionally subjecting the SV40 viruses or pseudo-viruses thus formed to digestion by nuclease to remove non-packaged DNA.

In the method of the invention, at least one other SV40 protein, preferably SV40 agnoprotein, can be added to the mixture of the SV40 capsid protein/s and the nucleic acid.

The DNA employed in the method of the invention can be DNA which encodes a therapeutic protein or peptide product which is not made or contained in mammalian

cells, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in such cells in abnormally low amount, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in such cells in defective form or is DNA which encodes a therapeutic protein or peptide product which is made or contained in such cells in physiologically abnormal or normal amount or is DNA which encodes a therapeutic RNA.

The therapeutic protein or peptide product can be an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

The nucleic acid employed in the method of the invention can alternatively be exogenous RNA, wherein said RNA is RNA which encodes a therapeutic protein or peptide product which is not made or contained in mammalian cells, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in such cells in abnormally low amount, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in such cells in defective form or is RNA which encodes a therapeutic protein or peptide product which is made or contained in such cells in physiologically abnormal or normal amount and wherein said RNA has regulatory elements, including translation signal, sufficient for the translation of said protein product in mammalian cells, operatively linked thereto.

The method of the invention can also be used for the *in vitro* construction of recombinant SV40 viruses or pseudoviruses comprising an exogenous protein or peptide comprising the steps of bringing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins into contact with the exogenous protein to give recombinant SV40 viruses or pseudoviruses; and optionally purifying the recombinant viruses or pseudoviruses thus obtained from any non-packaged protein.

In addition, the method of the invention can be used for the *in vitro* construction of SV40 pseudoviruses comprising exogenous antisense RNA, or ribozyme RNA or RNA or DNA which inhibits or prevents the expression of undesired protein/s in a mammalian cell, comprising the steps of bringing a semi-purified or pure SV40 capsid protein or a mixture of at least two such proteins into contact with the exogenous antisense RNA, or ribozyme RNA, or RNA or DNA which inhibits or prevents the expression of undesired protein/s in a mammalian cell, to give recombinant SV40 pseudoviruses; and optionally subjecting the SV40 pseudoviruses thus formed to digestion by nuclease to remove non-packaged DNA.

- 10 In a further aspect, the invention relates to mammalian cells infected with the constructs of the invention or with constructs obtained by any of the methods of the invention.

- Still further, the invention relates to a method of providing a therapeutic DNA, RNA, protein or peptide product or antisense RNA to a patient in need of such product by administering to the patient a therapeutically effective amount of the SV40 viruses or pseudoviruses of the invention or a therapeutically effective amount of infected cells according to the invention.

- Pharmaceutical compositions comprising as active ingredient a therapeutically effective amount of the SV40 viruses or pseudoviruses according to the invention or a therapeutically effective amount of infected cells according to the invention are also within scope of this application.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1(a) and 1(b): Self-Assembly of the SV40 capsid proteins

Nuclear extracts [Schreiber, E., *et al*, (1989) Nucl. Acids Res. 15:6419-6436] were prepared from Sf9 cells infected with the three recombinant baculoviruses expressing VP1, VP2 and VP3. Samples were adsorbed onto Formvar-carbon-coated copper grids and stained with 1% phosphotungstase, pH 7.2. The samples were viewed in a Philips CM-12 electron microscope, used at a voltage of 100kV, and photographed at a magnification of x60,000. The bar represents 50nm.

Fig. 1(a): Three fields of nuclear extracts of Sf9 cells infected with three recombinant baculoviruses, expressing VP1, VP2 and VP3.

Fig. 1(b): Wild type SV40, shown for comparison.

Figure 2(a)-2(c): Infectivity of SV40 virions and pSO3cat pseudovirions packaged *in vitro*.

Products of the *in vitro* packaging reaction were assayed for infectious units *in situ* hybridization, following infection of CMT4 monolayers.

Fig. 2(a): Autoradiograms showing infections centers produced by pSO3cat DNA packaged *in vitro*, using nuclear extracts of Sf9 cells infected either with recombinant baculovirus expressing VP1 or with the three recombinant viruses, as designated.

Fig. 2(b): Quantification of the results shown in Fig. 2(a).

Fig. 2(c): *In vitro* packaging of SV40 DNA, using nuclear extracts of Sf9 cells, uninfected or infected, as designated.

Figure 3(a)-3(d): Physical association of pSO3cat plasmid DNA with the capsid protein.

Fig. 3(a): The reaction products were placed on top of a 5-35% sucrose gradient in 10mM TrisHCl pH 7.4/150mM NaCl, with a 2M sucrose cushion, and centrifuged (SW 50.1 rotor) at 35000 rpm for 120 min., at 4°C. Fractions were collected from the bottom. Δ -SV40 capsid proteins, assayed by SDS-PAGE and western blotting with anti-VP1 antibody (Sandalon, Z., Herman-Dalyot, N., Oppenheim, A. B. And Oppenheim, A. Submitted for publication). \square - DNA was analyzed following treatment with 0.4M NaOH in 25mM EGTA and 20mM DTT at 37°C for 30 min., by electrophoresis on 1% agarose gels and Southern blotting, with pML2 as a probe. \circ -IU were assayed as described in Fig. 2.

Fig 3(b): Nuclear extracts of Sf9 cells infected with the three recombinant baculoviruses. The fractions were analyzed by SDS-PAGE as in (a).

Fig. 3(c): SV40 virions, analyzed as described for (a). Δ -SV40 capsid proteins; \square - SV40 DNA.

Fig. 3(d): pSO3cat DNA (1 μ g). The fractions were analyzed by electrophoresis on 1% agarose gels and EtdBr staining.

Figure 4(a) and 4(b): Expression of the transmitted DNA molecules.

Fig. 4(a): CMT4 cells, infected with *in vitro* packaged pSO3cat DNA, were assayed for CAT activity 3 days post infection. Essentially as previously described [Oppenheim. A., *et al.*, (1986) Proc. Natl. Acad. U.S.A. 83:6925-6929], for 60min. at 37°, using extracts of 10⁶ cells/ assay.

11

- 1 - No extract negative control;
- 2 - Mock infected CMT4 control;
- 3 - Control cells "infected" with pSO3cat DNA only;
- 4 - Cells infected with *in vitro* packaged pSO3cat; moi of 1-IU per 2×10^4 cells;
- 5 - Cells infected at a moi of 1-IU per 1×10^4 cells.

Fig. 4(b): Lysis of CV1 cells infected with *in vitro* packaged virions.

A - Mock infected;

B - "Infected" with DNA only;

C - Infected with *in vitro* packaged SV40.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to constructs capable of infecting a mammalian cell, comprising at least one semi-purified or pure SV40 capsid protein and a constituent selected from the group consisting of an exogenous DNA encoding an exogenous protein or peptide product, or encoding a therapeutic RNA, or itself a therapeutic product, a vector comprising exogenous DNA encoding an exogenous protein or peptide product, or encoding a therapeutic RNA, or itself a therapeutic product, an exogenous RNA encoding an exogenous protein or peptide product or itself a therapeutic product, a vector comprising an exogenous RNA encoding an exogenous protein or peptide product, or itself a therapeutic product, an exogenous protein or peptide product, and antisense RNA, ribozyme RNA or any RNA or DNA which inhibits or prevents the expression of undesired protein/s in a mammalian cell, and optionally further comprising operatively linked regulatory

elements sufficient for the expression and/or replication of said exogenous therapeutic RNA or of an exogenous protein or peptide in a mammalian cell.

The construct of the invention may optionally further comprise additional SV40 protein or proteins, preferably SV40 agnoprotein.

- 5 In specific embodiments, the constructs of the invention comprise a mixture of at least two semi-purified or pure SV40 capsid proteins.

In a further specific embodiment, the constructs of the invention comprise a mixture of three semi-purified or pure SV40 capsid proteins.

- 10 The SV40 capsid proteins of the invention can be semi-purified or pure VP1 or VP2 or VP3.

- 15 The constructs of the invention may comprise as said constituent an exogenous circular or linear DNA encoding an exogenous protein or peptide product, or itself a therapeutic product, or encoding therapeutic RNA, or a vector comprising exogenous DNA encoding therapeutic RNA or encoding an exogenous protein or peptide product. Delivery into cells of linear DNA, by infecting the cells with constructs of the invention comprising such linear DNA, may be advantageous for recombination, i.e. integration into the cellular genome for stable expression.

- 20 Specifically, said DNA is DNA which encodes a therapeutic protein product or is itself a therapeutic product which is not made or contained in said cell, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is DNA which encodes a therapeutic protein or peptide which is made or contained in said cell in physiologically abnormal or normal amount or encodes a
25 therapeutic RNA.

The therapeutic protein or peptide product can be any protein of interest, such as an enzyme, a receptor, a structural protein, a regulatory protein or a hormone. Of particular interest are proteins which are missing or defective in patients suffering genetic disorders. A specific example may be β -globin, missing in patients with β -thalassemia.

The constructs of the invention may optionally comprise SV40-derived *ori* DNA sequence as said replication regulatory element. The exogenous DNA may optionally have, operatively linked thereto, additional DNA sequence/s encoding one or more regulatory elements sufficient for the expression of the exogenous protein or peptide encoded thereby in mammalian cells.

In an additional aspect, in constructs of the invention said constituent is exogenous RNA, particularly RNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount, said RNA having regulatory elements, including translation signal/s sufficient for the translation of said protein or peptide product in said mammalian cell, operatively linked thereto.

As in the embodiments containing RNA, the therapeutic protein or peptide product encoded by the exogenous RNA may be any protein of interest, such as an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

Packaging of RNA may be advantageous for "short term", transient gene activity. Packaging of RNA in SV40 pseudovirions, instead of, or in addition to DNA, will allow delivery of mRNA into mammalian cells. The mRNA should include

mammalian translation signal, for example Kozak sequences. Such constructs will facilitate transient production of proteins, having high specific function, *in vivo*.

The constructs of the invention will also enable the delivery of ribozyme RNA, which can be used in any application where specific RNA cleavage is desired, as an
5 anti-AIDS agent or as an agent against other viral infections or for other therapeutic purposes.

A specific example may be chronic myelogenous leukemia (CML). CML is a clonal stem-cell disorder which accounts for about 25 percent of all leukemias, with an annual incidence of one per 100,000 population, affecting all age groups, with a
10 peak incidence in the fifth and sixth decades of life. Clinically, CML is characterized by a triphasic course. The initial chronic phase, often develops insidiously and is marked by an increased pool of committed myeloid progenitor cells. After a few weeks to several years (median duration 42 months) the disease turns into a phase of "acceleration", which later progresses to the acute phase. The
15 median survival, from diagnosis, in the acute phase is approximately 4 months.

CML is genetically characterized by the presence of the Philadelphia chromosome (Ph⁺), which is the result of reciprocal translocation between chromosomes 9 and 22. At the molecular level, the proto-oncogene *abl* from chromosome 9 is translocated to the breakpoint cluster region (*bcr*) on chromosome 22, creating two
20 major types of junctions: L-6 and K-28, each resulting in the formation of *bcr/abl* hybrid gene, expressing a fusion protein of 210kd, which causes the disease. Possible use of antisense (18-mer) oligonucleotides, or DNA encoding antisense RNA, directed against the expression of the *bcr/abl* gene as tumor specific agents which alter the transformed phenotype of leukemia cells cultured *in vitro* has
25 already been demonstrated [Szczylik, C. *et al.* (1991). *Science*, **253**:562-565; Garcia-Hernandez, B. & Sanchez-Garcia, I. (1996) *Mol. Medicine*, **2**:1076-1551].

Therefore, the constructs according to the invention may comprise as said constituent antisense RNA or DNA encoding antisense or ribozyme RNA, or any RNA or DNA which inhibits or prevents the expression of undesired protein/s or peptide/s in mammalian cells.

- 5 In this specific embodiment, said antisense RNA or DNA encoding antisense RNA can be directed against the expression of the *bcr/abl* transcript, or against an HIV transcript.

In additional embodiments, the constructs of the invention may comprise as said constituent an exogenous protein or peptide product.

- 10 In preferred such constructs, said constituent is exogenous protein or peptide product is, respectively, a therapeutic protein or peptide product which is not made or contained in said cell, or is a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is a therapeutic protein or peptide product which is made or contained in said cell in defective form or is a
15 therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount.

- The delivery of packaged proteins or peptides will also facilitate their transient function *in vivo*. This approach will be used when long term effects of the packaged protein are not required or may be dangerous. Thus, for example, the delivery of
20 packaged proteins may be useful in cases where transient local production of appropriate growth factors, for example, FGF (Fibroblast Growth Factor) is required, to accelerate internal wound healing or post-operative incision healing. Local transient introduction of blood clotting factors may be desirable for prevention of hemorrhage and introduction of anti-coagulating factors may be
25 desirable for dissolving unwanted blood clots. Application of infecting pseudo-virions on site may be by catheters or any other suitable physical means.

Some proteins may have specific function on the fate of DNA delivery. The constructs of the invention will enable the delivery of mRNA encoding for a protein which promotes homologous recombination, or the delivery of such protein itself. Pseudovirions carrying a gene will be used in co-infection, together with constructs
5 comprising as said constituent mRNA coding for proteins which promote homologous recombination such as REC A, or construct comprising as a constituent such protein/s. This technique will enable gene replacement therapy.

The constructs of the invention are capable of infecting mammalian, particularly human cells. Specific cells may be hemopoietic cells, such bone marrow cells,
10 peripheral blood cells and cord blood cells, or liver cells, epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells, tumor cells, nerve cells and germ line cells.

In another aspect, the invention relates to a method for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid, comprising the
15 steps of bringing a semi-purified or pure SV40 capsid protein or a mixture of at least two such proteins into contact with said exogenous nucleic acid to give recombinant SV40 viruses or with a vector comprising said exogenous nucleic acid to give pseudoviruses; and optionally subjecting the SV40 viruses or pseudoviruses thus formed to digestion by nuclease to remove non-packaged DNA.

20 The SV40 capsid protein are preferably semi-purified or pure SV40 VP1, VP2 or VP3.

The method of the invention may employ, in addition to said semi-purified or pure SV40 capsid protein/s and said nucleic acid, at least one other SV40 protein, preferably SV40 agnoprotein.

25 The exogenous nucleic acid is preferably circular or linear DNA or is RNA. The exogenous nucleic acid preferably encodes a therapeutic protein or peptide product or is itself therapeutic product.

In specific embodiments, said DNA or RNA are DNA or RNA which encode a therapeutic protein or peptide product which is not made or contained in said cell, or which encode a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or which encode a therapeutic protein or peptide product which is made or contained in said cell in defective form or which encode a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount or is a DNA which encodes a therapeutic RNA.

Said exogenous DNA or RNA preferably encode a therapeutic protein or peptide product which is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

In the method of the invention, SV40-derived *ori* DNA sequence may be added and said exogenous nucleic acid optionally has DNA sequence encoding one or more regulatory elements, sufficient for the expression of said exogenous protein or peptide in said mammalian cell, operatively linked thereto.

When said nucleic acid is exogenous RNA, it has to have the necessary regulatory signals, including a translation signal, sufficient for the translation of said protein or peptide product in a mammalian cell, operatively linked thereto.

In a further embodiment, the invention relates to a method for the *in vitro* construction of recombinant SV40 viruses or pseudoviruses comprising as said constituent an exogenous protein or peptide comprising the steps of bringing a semi-purified or pure SV40 capsid protein or a mixture of at least two such proteins into contact with an exogenous protein or peptide, to give recombinant SV40 viruses or pseudoviruses, and optionally purifying the recombinant viruses or pseudoviruses thus obtained from any non-packaged protein.

In this embodiment, the exogenous protein or peptide can be, respectively, a naturally occurring or recombinant protein or peptide, a chemically modified protein or peptide, or a synthetic protein or peptide.

The exogenous protein or peptide product are, respectively, a therapeutic protein or peptide product not made or contained in a mammalian cell, or are a therapeutic protein or peptide product made or contained in said cell in abnormally low amount or are a therapeutic protein or peptide product made or contained in said cell in defective form or are a therapeutic protein or peptide product made or contained in said cell in physiologically abnormal or normal amount.

10 In addition, the method of the invention can be used for the *in vitro* construction of SV40 pseudoviruses comprising antisense RNA, or exogenous DNA encoding antisense RNA, or ribozyme RNA, or RNA or DNA which inhibits or prevents the expression of undesired protein/s or peptide/s in a mammalian cell, comprising the steps of bringing a semi-purified or pure SV40 capsid protein or a mixture of at
15 least two such proteins into contact with the exogenous antisense RNA or exogenous DNA encoding antisense RNA, or ribozyme RNA, or RNA or DNA which inhibits or prevents the expression of undesired protein/s or peptide/s in a mammalian cell, to give recombinant SV40 pseudoviruses; and optionally subjecting the SV40 pseudoviruses thus formed to digestion by nuclease to remove
20 non-packaged DNA.

In this embodiment, the said antisense RNA or DNA encoding antisense RNA can be directed against the expression of *bcr/abl* transcripts, or against an HIV transcripts.

The method of the invention is suitable for the preparation of constructs which are
25 capable of infecting any suitable mammalian cell. Specific cells are hemopoietic cells, such as bone marrow cell, peripheral blood cells and cord blood cells, or liver

cells, epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells, tumor cells, nerve cells and germ line cells.

In yet a further aspect, the invention relates to a mammalian, preferably human cell infected with any of the constructs of the invention, or constructs obtained by any of the methods of the invention.

Still further, the invention concerns a method of providing a therapeutic DNA, RNA, protein or peptide product or antisense RNA or DNA encoding antisense to a patient in need of such product by administering to said patient a therapeutically effective amount of any of the said SV40 viruses or pseudoviruses or a therapeutically effective amount of said infected cells.

The invention also relates to pharmaceutical compositions comprising as active ingredient a therapeutically effective amount of the SV40 virus or pseudoviruses of the invention or a therapeutically effective amount of the infected cells of the invention.

The constructs of the invention are very efficient in gene transfer into a variety of cells, including human hemopoietic cells and probably also stem cells. Thus, they may be suitable for treating a wide spectrum of diseases. Plasmids carrying the desired gene and the SV40 *ori* and, optionally *ses*, are encapsidated in COS cells, optionally with helpers, as SV40 pseudovirions, and transmitted into the target cells by viral infection. The prokaryotic DNA is removed after propagation in bacteria and before encapsidation. The constructs include only 200bp of SV40 DNA, with cloning capacity of over 5kb. Thus plasmids carrying over 95% human DNA are efficiently transferred into human hemopoietic cells.

The invention provides for safer and cheaper products for medical use, which may be prepared under aseptic conditions. A major advantage is that proteins are readily made in insect cells. While semi-purified proteins (nuclear extracts) are exemplified, purified proteins can be employed. Further, DNA is prepared in

bacteria and can be purified before it is packaged. This ensures high purity and high quality DNA minimizing the chance for picking spontaneous mutations and/or rearrangements. In addition, *in vivo* pseudovirions are present in a solution which also contains constituents of the cells in which they were grown. In contrast, infection by retroviral vectors prepared *in vivo* is done by co-culturing of the patients cells with the producer cell-lines (usually murine). Although *in vivo* prepared viral sectors (such as adenoviruses or adeno-associated virus) can be purified, this may substantially increase production cost, as purification is also associated with loss of virion particles. In addition, in helper-free packaging cell-lines (of any virus) there is always a risk of contamination by recombinants. These could be either the wild-type virus or unknown recombination products, carrying potentially harmful (cellular) genes. The risk is completely abolished when packaging is done *in vitro*. Moreover, *in vitro* packaging can accommodate larger plasmids than *in vivo* packaged SV40 pseudovirions: The *in vivo* packaged pseudovirions accommodate only up to about 5.4kb of DNA [Oppenheim, A. & Peleg, A. (1989) Gene 77:79-86]. In the present *in vitro* method about 7.5kb have been packaged successfully, and larger plasmids can be packaged. Regulatory elements (e.g. β -globin, LCR), which interfere with packaging *in vivo*, are not expected to interfere with packaging *in vitro*. An additional important advantage is that the *ses* element is not required for *in vitro* packaging (Tables 2 and 3), reducing the size of the required SV40 sequences to about 100bp, comprising the *ori*, in the exemplified experiments. Embodiments without even this element are also contemplated. In the present examples, the *ori* element was required for the assay of infectious units. The high flexibility afforded by the method and constructs of the invention may allow the development of gene targeting (or gene replacement therapy).

EXAMPLES

Cloning the genes of the SV40 capsid proteins for expression in bacteria

First, plasmids designed to express the complete VP1, VP2 and VP3 polypeptides as fusion proteins to glutathion-S-transferase (GST) in *E. coli* [Smith, D.B. & Johnson, K.S. (1988) *Gene* 67:31-40]. The respective SV40 fragments were cloned into the
5 vector with the aid of PCR. Expression level was high, leading to the production of insoluble inclusion bodies. Similarly, it was recently reported [Clever *et al.*, *ibid.*] that a truncated VP2 fused to GST also yielded an insoluble product.

Preparation of antibodies

10 The three GST-fusion capsid proteins were used to raise polyclonal antibodies in rabbits. Antibodies against GST-VP1 did not cross-react with VP2 and VP3. As expected, antibodies against GST-VP2 reacted both with VP2 and VP3, and did not cross-react with VP1.

Cloning the SV40 late genes for expression in insect cells

15 SV40 DNA fragments were cloned into the plasmid vectors pVL1393 and pVL1392 (commercially available from PharMingen, San Diego, California), derived from *Autographa californica* nuclear polyhedrosis virus (AcMNPV) [Luckow, V.A. & Summers, M.D. (1988) 6:47-55; Luckow, V.A. & Summers, M.D. (1989) *Virology* 170:31-39] In these vectors expression of the foreign gene is driven by the strong
20 promoter for the viral occlusion protein, polyhedrin. The genes for the capsid proteins were cloned into pVL1393 as follows: VP1 was cloned by introducing a *StuI-BclI* DNA fragment (SV40 coordinates 1463-2770) into the plasmid cleaved by restriction endonucleases *SmaI* and *BglII*. The VP2 gene was cloned by ligating a *HincII-EcoRI* fragment (522-1782) between the *SmaI* and *EcoRI* sites. The VP3
25 gene, which is nested in the VP2 gene (it is translated from an internal AUG signal), was cloned by using a *Sau3AI-EcoRI* fragment (874-1782) and the *BamHI* and

EcoRI sites of pVL1393. A fourth late polypeptide, the agnoprotein (or LP1), encoded by the leader region of the late 16S mRNA, appears to play a role in expediting virion assembly [Carswell, S. & Alwine, J.C (1986) *J. Virol.* **60**:1055-1061; Resnick, J. & Shenk, T. (1986) *J. Virol.* **60**:1098-1106] The agnogene, a
5 *PvuII-MboI* fragment (273-873), was cloned between the *SmaI* and *BamHI* sites of plasmid pVL1392.

The structures of the four recombinant plasmids were confirmed by restriction analysis and after propagation in *E. coli*. Sequence analysis was performed for The recombinant plasmids carrying VP2 and VP3. Recombinant baculovirus carrying
10 the four respective genes were produced using the BaculoGold kit (kindly provided by PharMingen, California). The technique relies on homologous recombination between the plasmid and a modified type of baculovirus with a lethal deletion. Each of the recombinant plasmids was co-transfected, together with linearized DNA of the defective baculovirus, into *Spodoptera frugiperda* (Sf9) cells. Virus was
15 harvested 4 days later, according to the protocol supplied by the manufacturer. To obtain high titer stocks each of the recombinant virus was amplified by 3 cycles of infection (5 cycles for the VP2 recombinant virus) of freshly seeded Sf9 cells [Summers, M.D. & Smith, G.E. (1988) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station,
20 College Station, Texas] The final titers of the 4 recombinant baculoviruses stocks were $2-4 \times 10^8$ pfu/ml.

The SV40 proteins were produced in Sf9 cells, infected at a multiplicity of 10 pfu/cell and grown at 27°C. Cells were harvested and the soluble proteins were analyzed by SDS-PAGE [Laemmli, E.K. (1970) *Nature* **227**:80-685] and Western
25 blotting [Harlow, E. & Lane, D. (1988) Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, N.Y., Cold Spring Harbor] using antibodies raised against the corresponding GST-fusion proteins. Kinetic studies showed increasing levels of the SV40 proteins from 3 to 6 days postinfection.

The three capsid proteins assemble spontaneously to form SV40-like particles

The SV40 capsid proteins were produced, separately, in *Spodoptera frugiperda* (SF9) cells from recombinant baculovirus each carrying the genes coding for VP1, VP2 or VP3. The cells were harvested and nuclear and cytoplasmic fractions were
5 analyzed by SDS-PAGE and western blotting. The results demonstrated that VP1 and VP3 were preferentially present in the nuclear fraction, whereas VP2 was preferentially cytoplasmic (not shown). When the three proteins were co-expressed in the same cells (following infection with the three recombinant baculoviruses together), all three proteins were present in the nuclear fraction. The capsid proteins
10 self-assembled to form SV40-like structures of various sizes. Electron microscopy of nuclear extracts of the infected cells showed abundance of "empty" SV40-like capsids and heterogeneous aggregates of variable size, mostly 20-45 nm (Fig. 1(a)). Under the same staining conditions wild type SV40 virions are 45 nm with well defined boundaries (Fig. 1(b)). Occasionally smaller, presumable empty or defective
15 particles, are also seen in wild type SV40 stocks.

To purify VP1, nuclear extracts of infected cells were placed on 15-35% glycerol gradient with a 2M sucrose cushion. Western blot analysis and EM studies demonstrated that the majority of VP1 was present as high MW structures in 2 peaks, one at the cushion and the other at the bottom of the tube. About a third of
20 the protein was present as pentamers (10S units; MW ~210kd). In most of the experiments, monomers were not seen. The results indicate that VP1 molecules readily form pentamers and high MW structures at this high concentration. VP1 pentamers and higher MW structures are stabilized by S-S bonds [Gharakhanian, E. *et al.* (1995) *Virology* **207**:251-254] and by Ca^{++} [Liddington *et al.*, *ibid.*]. It appears
25 that only at very low concentrations VP1 molecules may remain monomeric [Gharakhanian *et al.*, *ibid.*].

Similar experiments, performed with nuclear extracts of cells containing the 3 capsid proteins, demonstrated that the three proteins co-precipitated together in the

glycerol gradients in two high MW fractions, at the very bottom of the tube (fraction I) and at the cushion (fraction II). In addition, peaks corresponding to VP1 pentamers and to VP2 monomers were also seen.

In SV40, the three capsid proteins are expressed from a single promoter with complex regulatory controls, including several transcription start sites, alternative splicing to two major species, 16S RNA, producing the agnoprotein (which is not part of the capsid) and VP1 and 19S, producing VP2 and VP3. The two bicistronic messages contain internal translation initiation signals. This organization is thought to facilitate coordinated expression at the correct ratio for packaging (Sedman, S.A., *et al.* (1989) *J. Virol.* 63:3884-3893; Sedman, S.A. *et al.* (1990) *J. Virol.* 64:453-457]. Co-production of the capsid proteins VP1, VP2 and VP3 in Sf9 cells was performed by infecting with the 3 baculovirus species at equal multiplicities. Nevertheless, the ratio of the 3 proteins in fraction II was similar to the ratio obtained in monkey CMT4 cells [Gerard, R.D. & Gluzman, Y. (1985) *Mol. Cell. Biol.* 5:3231-3240] infected with wild type SV40. This was not true for fraction I. Furthermore, EM studies revealed that fraction II was relatively homogeneous, containing particles of approximately SV40 size, which appeared "empty", as they allow penetration of the stain. On the other hand the particles in fraction I were highly heterogeneous in size.

DNA is packaged in the SV40 capsids in vitro, forming infectious particles

A. Packaging experiments were performed with SV40 DNA and with heterologous plasmid DNA. Nuclear extracts of Sf9 cells were prepared according to Schreiber [Schreiber, (1989) *ibid*]. 2 μ l of nuclear extracts (protein concentration 1-2 μ g/ μ l) were mixed by vortex with 1 μ g DNA in a total volume of 4 μ l and placed at 37°C for 6hr. Preliminary experiments showed that shorter incubation periods (1-4hr) gave lower yields of infectious particles. CaCl₂ and MgCl₂ were added to final concentrations of 100 μ M and 8mM respectively, to a total volume of 6 μ l, and the reactions were incubated for an additional 1hr on ice. DNase I digestion was

performed using 0.5 unit of enzyme for 10min on ice, and stopped by the addition of EDTA to a final concentration of 5mM.

The DNase I treatment was used to remove DNA which was not stably packaged. The reaction products were assayed for infectious units (IU) on CMT4 monolayers, grown in Dulbecco's modified Eagle's medium with 10% FBS, using a standard SV40 infection protocol. CMT4 are permissive African green monkey kidney cells that harbor the gene for SV40 T-antigen expressed from the inducible metallothionein promoter [Gerard, R.D. & Gluzman, Y. (1985) Mol. Cell. Biol. 5:3231-3240]. Sub-confluent monolayers were incubated with the packaging mixture for 120 min at 37°C, with occasional agitation, followed by the addition of fresh medium containing 0.1mM ZnCl₂ and 1μM CdSO₄ for the induction of T-antigen expression. Infective centers were scored by in situ hybridization. The number of infective centers obtained for a 6μl reaction mixture was used in computing the titer of IU/ml (Fig. 2(a)).

The procedure yielded infectious units both with SV40 DNA (Fig 2(c)) and with pSO3cat DNA (Figs. 2(a) and 2(b)). A typical experiment, demonstrating infectivity of *in vitro* packaged pSO3cat, is shown in Fig. 2(a). Under these conditions naked DNA sometimes also entered the cells. However, the DNase I treatment completely removed the naked DNA background from the assay. The experiments showed that using the nuclear extracts containing VP1+VP2+VP3 directly, without purification on glycerol gradients, yielded 90 infectious centers, per 6μl packaging reaction, equivalent to 1.5×10^4 infective units IU per ml. Pre-treatment before the infection with DNase I reduced the number to 77 (1.2×10^4 IU/ml). Incubation with nuclear extracts containing VP1 alone produced 75 infectious centers (1.2×10^4 IU/ml), almost the same as with the three capsid proteins. However, The pre-treatment with DNase reduced the number almost 2 fold, to 40 (a titer of 6.6×10^3 IU/ml). This was seen both for pSO3cat DNA (Fig. 2(b)) and for SV40 DNA (Fig. 2(c)), suggesting that the DNA in those particles was not as effectively protected from DNase I, under

the conditions described below. These results suggest that VP2 and VP3 contribute to the stability of the DNA-capsid complexes. In the absence of DNase I treatment naked DNA was also sometimes infectious. However, DNase I digestion completely removed the naked background from the assay. Therefore, all subsequent experiments included DNase I digestion. Fig. 2(c) also demonstrates that presence of the SV40 capsid proteins is required for the production of infectious particles, as “packaging” with nuclear extracts of uninfected Sf9 gave negative results. Few “infectious units” were seen in cells treated with SV40 DNA only, demonstrating the ability of naked DNA to penetrate cells. DNase I treatment completely removed this background.

Insect cells were infected with three recombinant baculoviruses encoding for the three capsid proteins, VP1, VP2 and VP3, at moi 10 each. After 4 days nuclear extracts were prepared essentially as described by Schreiber [Schreiber, E., *et al.* (1989) *Nucleic Acids Res.* **15**:6419-6436] by shaking the nuclei, isolated with 10% NP-40, in a buffer containing 20mM HEPES pH 7.9, 0.4M NaCl, 1mM EDTA, PMSF and leupeptin were added just before use. The nuclei from each 75x cm² culture bottle were extracted with 150μl buffer.

The nuclear extract was used in packaging experiments performed exactly as described above. For each packaging reaction 2μl nuclear extracts were mixed with 1μg DNA (either SV40 or pSO3cat) in a total volume of 4μl, for several hours at 37°C. The reaction mixture was transferred to ice, 2μl of 0.4mM CaCl₂ were added and incubated for 60 min on ice. This was followed by the addition of 2μl containing 0.5 unit of DNase I, and the incubation continued for 10 min. on ice. The reaction was stopped by the addition of 2μl of 25mM EDTA (final concentration 5mM). Serum-free medium was added (300μl) and the mixture was applied to a sub-confluent CMT4 culture in a 6cm diameter culture plate. The cultures were incubated at 37°C with gentle agitation every 20 min. Two hours later the infection mixture was sucked off and 5ml fresh medium containing 5% FBS was added. After

about 40hr, to allow replication of the DNA transmitted by the infectious particles, the monolayers were transferred to nitrocellulose layers and processed for hybridization.

B. In another study, the inventors investigated the properties of the self-assembled empty capsids shown in Fig. 1 and Fig. 3(b). It was found that DTT and EGTA induced dissociation of the self-assembled empty capsids. Therefore, an alternative protocol was devised, in which the proteins are pre-treated with DTT and EGTA prior to their incubation with the DNA. The DTT and EGTA are dialyzed out and replaced by Ca^{++} ions. Nuclear extracts of Sf9 were prepared as described above [Shreiber *et al.* (1989), *ibid.*] from cells infected with the three recombinant baculoviruses expressing VP1, VP2 and VP3 at multiplicity of infection (moi) 10 for each recombinant baculovirus. For each packaging reaction, 10 μ l of nuclear extracts, containing 10-15 μ g protein, are incubated with DTT at a final concentration of 10mM for 5 min at 33°C, with gentle shaking, to allow the capsid proteins to dissociate. The reactions are then cooled on ice, 1 μ g of DNA is added to each reaction and the ingredients are thoroughly mixed by vortexing and incubated on ice for 30min. Total volume for each reaction is 20 μ l. The reaction mixtures are then dialyzed against a buffer containing 10mM CaCl_2 , 150mM NaCl, 10mM Tris-HCl pH 7.2 for 24hr in the cold, with two changes.

This protocol yielded, in 4 different experiments, a titer of $1-2.5 \times 10^4$ IU/ml, which is similar to the titer obtained in the above described protocol A.

Physical association between capsids and DNA

To obtain physical evidence for the association of DNA with capsid particles, the reaction products were sedimented in a sucrose gradient. To prevent the action of endonucleases present in the nuclear extract, EDTA was added to a final concentration of 4mM. Under these conditions, non-packed DNA, which sedimented at the top of the gradient (as does free plasmid DNA, Fig.3(d)),

remained intact and supercoiled, as was evident from agarose gel electrophoresis. The majority of the capsid proteins to the sucrose cushion, similarly to authentic SV40 (Figs. 3(a) and 3(c)). A small portion of the DNA (pSO3cat) co-sedimented with the capsid proteins to the sucrose cushion. That DNA was visible on agarose gel electrophoresis and Southern blotting only after the particles were dissociated by treatment with EGTA and DTT at alkaline pH, suggests that it had been contained within particles. The DNA in those fractions migrated on the gel as did supercoiled pSO3cat DNA (not shown), indicating the presence of intact plasmid molecules. Analyses of the fractions that contained both capsid proteins and DNA (Fig. 3(a) fractions 2-4) demonstrated the presence of infectious units. The results taken together indicate the formation of capsid particles which contain supercoiled plasmid DNA and which are infective.

Proteins present in nuclear extracts assist in packaging in vitro

SV40 assembly requires precise insertion of the carboxy-terminal arms of the VP1 molecules into the neighboring pentamers. However, an arm can easily insert, instead, into its own pentamer, thus interfering with assembly. To explain how such mistakes are avoided, the participation of chaperones has been invoked [Liddington, R.D, *et al.* (1991) *ibid.*]. The inventors rationalized that such chaperones may also be present in nuclear extracts of the Sf9 cells, and that they may facilitate capsid formation and entry of DNA into pre-formed aggregates. Indeed, experiments with crude nuclear extracts consistently yielded approximately 10 fold more infectious units than those performed with capsid aggregates which had been partially purified by glycerol gradients (Table 2). These results suggest that additional proteins present in the nuclear extract of Sf9 cells, presumably, chaperons, enhance DNA packaging in vitro. As chaperones require ATP for their activity we investigated whether the addition of ATP to the reaction improves packaging. Packaging was performed as above, in the presence of 5mM ATP. The results shown in Table 1

suggest that ATP may indeed improve *in vitro* packaging, although the titers obtained in these experiments were lower than the usual.

The inventors have recently started to investigate whether the agnoprotein can also enhance DNA packaging *in vitro*. Nuclear extracts of Sf9 were prepared as described above from cells infected with the four recombinant baculoviruses expressing VP1, VP2 and VP3 and the agnoprotein, at moi 10 for each recombinant baculovirus. Packaging of DNA was performed after treatment of the nuclear extracts with DTT, as described above for the nuclear extract which contained the three capsid proteins. The results presented in Table 1, although inconclusive, suggest that the agnoprotein may improve packaging. The effect of the agnoprotein on packaging may be more critical when purified capsid proteins are used in the packaging reaction.

Table 1.

The effect of ATP on *in vitro* packaging of pSO3cat

Capsid proteins	Infectious units/ml*	
	No ATP added	+ ATP
Nuclear extracts containing VP1+VP2+VP3	2.7×10^3	8×10^3
Nuclear extracts containing VP1+VP2+VP3+agnoprotein	3.8×10^3	1.4×10^4

*titers after DNase I treatment

The infectious particles transmit complete DNA molecules which are biologically functional

The results shown in Fig. 2(c) may presumably reflect DNA which did not penetrate the cells but remained adsorbed on the cell surface, in structures which are DNase I resistant. To prove that the *in vitro* packaged DNA entered the cells, and that the DNA is biologically active in gene expression, the inventors asked whether the *cat* gene transmitted by pSO3*cat* expressed the CAT enzyme. CMT4 cells were harvested 3 days postinfection with *in vitro* packaged pSO3*cat*. CAT assays [Oppenheim *et al.* (1986) *ibid*] demonstrated enzyme activity at a significant level (Fig. 4(a)), although the moi in this experiment was very low (less than $1 \text{ IU} \times 10^4$ cells). These results indicate that the infectious units transmitted biologically active DNA into the target cells.

It was then to be verified whether complete DNA molecules became packaged in this experimental protocol. For production of SV40 virions, the complete SV40 molecule is required, including the regulatory region, and the early and the late genes. SV40 DNA was packaged as described above and used to infect CV-1 cells at a low moi ($\sim 1 \text{ IU} / 10^4$ cells). After 2 weeks extensive cell lysis was visible, indicating that productive SV40 infection was going on (Fig. 4(b)). Only complete SV40 DNA, which can produce functional T-antigen as well as the late proteins, can produce virions on CV-1 cells. It was therefore concluded that the *in vitro* packaging system described herein produces particles which contain the complete circular DNA molecules.

In vitro packaging is not dependent on ses

A small DNA element, *ses*, is required for SV40 assembly *in vivo*. *ses* appears to play multiple roles in packaging. It is probably a recognition site for the capsid proteins, serving as a nucleation center in the initiations of viral assembly [Dalyot-Herman *et al.*, *ibid*]. In addition, *ses* functions in regulating the late stages of the

SV40 life cycle, in turning off viral gene activity and by allowing the capsid proteins to induce nucleosomal reorganization and chromatin condensation in the transition from replication and late transcription to packaging [Oppenheim *et al.* (1992) *ibid.*]. *In vitro*, the use of non-transcribing, naked plasmid DNA may be predicted to circumvent part of the requirements for *ses*. As shown in Tables 1 and 2, *ses*⁺ (pSOγ-S) and *ses*⁻ (pSOγ-N) plasmids are indeed packed *in vitro* at similar efficiencies, indicating that *ses* is dispensable for *in vitro* packaging. Possibly, *in vitro* packaging of SV40 pseudovirions may allow efficient transfer of human genes without any accessory viral DNA.

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Table 2

Packaging with purified capsid proteins in comparison with total nuclear extracts of Sf9 cells infected with recombinant baculovirus.

Capsid protein	Infectious units/ml			
	SV40	pSO3cat	pSOγS	pSOγN
Nuclear extract containing VP1+VP2+VP3	1.5x10 ⁴	4x10 ⁴	7.5x10 ⁴	9.5x10 ⁴
Purified ^a VP1+VP2+VP3	1.7x10 ³	2.6x10 ³	<1.7x10 ³	1.6x10 ³
Nuclear extract containing VP1	7.5x10 ³	5.3x10 ³	N.D.	N.D.
Purified ^a VP1	6.7x10 ³	<1.7x10 ²	<1.7 x10 ²	5 x10 ²
Nuclear extract of uninfected SF9 cells	0	0	N.D.	N.D.

^apartially purified by glycerol gradient

Plasmid significantly larger than SV40 can be packaged in vitro

The genome size of SV40 plasmid is 5,243bp, and the upper limit of plasmid packaging is ~5.4-5.7 kb [Chang, X.B. & Wilson, J.H. (1986) *J. Virol.* **58**:393-401; Dalyot, N. (1994) Regulation of human globin genes and the development of a model for gene therapy of β -thalassemia, Ph.D. Thesis, The Hebrew University, Jerusalem]. SV40 DNA is packed *in vivo* as a minichromosome, complexed in nucleosomes, occupying large space within the virion particle [Martin, R.G. (1977) *Virology* **83**:433-437]. Further experiments were performed under the same conditions as before, except that instead of SV40 or pSO3cat DNA other plasmids, significantly larger than SV40 size, were used. In each experiments, 1 μ g of plasmid DNA was incubated with 2 μ l of nuclear extracts of Sf9 cells infected with VP1+VP2+VP3. The experiments were repeated at least twice for each plasmid. Typical results are shown in Table 3.

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Table 3

In Vitro Packaging of Various Plasmid

Plasmid units	Properties	Size(kb)	Infectious units/ml
SV40		5.2	4.2x10 ⁴
pSO3cat		4.1	2.4x10 ⁴
pSOγ-S ^a	<i>ses</i> ⁺	4.3	7.5x10 ³
pSOγ-N ^a	<i>ses</i> ⁻	4.2	9.5x10 ³
pSO6β-1 ^b	carries β-globin	7.3	3.3x10 ⁴
pSO6β-5 ^b	carries β-globin+LCR element	7.4	1.2x10 ⁴
pSO6β-9 ^b	carries β-globin+LCR element	7.0	1.2x10 ⁴
pSM1 ^c	carries MDR1	7.1	7.3x10 ³

^a described in Oppenheim, A., *ibid.*

^b described in Dalyot, N., Ph.D Thesis (1996) The Hebrew University, Jerusalem

5 ^c described in ,e.g., WO95/30762.

Importantly, the experiments demonstrated that various plasmids, carrying useful genes, can be packaged. Furthermore, packaging *in vitro* is not limited to 5.4-5.7kb of plasmid DNA, and plasmids over 7kb can be almost as efficiently packaged. This is presumably because under *in vitro* conditions naked DNA is packaged, rather than a minichromosome (which includes the DNA complexes in nucleosomes). The minichromosome occupies much more space, as compared to DNA of the same size, within the pseudoviral particle. It is also possible that in the absence of histones, the internal capsid proteins VP2 and VP3 assist in plasmid DNA condensation. The

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titers obtained in these experiments, around $1-5 \times 10^4$ IU/ml, are comparable to titers obtained by *in vitro* packaging of a number of currently used vectors.

In vitro packaging is probably accomplished by a mechanism which is different from the packaging *in vivo*. In the latter process, the viral capsid proteins are thought to assemble around the viral minichromosome, while *in vitro*, empty capsid-like structure (Fig. 1) serve as starting material. Furthermore, *in vitro* packaging utilizes naked DNA, prepared in *E. coli*. This result, combined with many of the inventors' previous studies on *in vivo* packaging, leads to the prediction that potent regulatory sequences, such as β -globin LCR, which interfere with viral packaging *in vivo* [Chang *et al.* (1992) *ibid*; Dalyot *et al.* (1994) *ibid.*], will not interfere with *in vitro* packaging. It is hypothesized that the LCR elements interfere with *in vivo* packaging of SV40 pseudovirions by the formation of higher order nucleoprotein structures which is not compatible with chromatin condensation [Dalyot, N. (1994) *ibid.*]. The use of supercoiled plasmid DNA, in the absence of regulatory proteins which bind to these regulatory elements, is predicted to relieve this problem *in vitro*. Indeed, two LCR containing plasmids, pSO6 β -5 and pSO6 β -9, which are poorly packed *in vivo* (producing titers of 10^3 and $\sim 10^4$ IU/ml, respectively [Dalyot, N. (1994) *ibid.*], yield here titers similar to pSO3cat (Table 3). The present results suggest that *in vitro* packaging will allow to combine in the constructs for gene therapy the optimal regulatory signal, which will lead to important improvement in expression of the delivered genes.

CLAIMS:

1. A construct capable of infecting a mammalian cell comprising
at least one semi-purified or pure SV40 capsid protein; and
5 a constituent selected from the group consisting of
 - an exogenous DNA encoding an exogenous protein or peptide product, or encoding therapeutic RNA, or itself a therapeutic product,
 - a vector comprising an exogenous DNA encoding an exogenous protein or peptide product, or encoding therapeutic RNA, or itself a therapeutic product,
10
 - an exogenous RNA encoding an exogenous protein or peptide product or itself a therapeutic product,
 - a vector comprising an exogenous RNA encoding an exogenous protein or peptide product or itself a therapeutic product,
 - 15 - an exogenous protein or peptide product, and
 - antisense RNA, ribozyme RNA or any RNA or DNA which inhibits or prevents the expression of undesired protein/s in said mammalian cell; andoptionally further comprising operatively linked regulatory elements sufficient for the expression and/or replication of said exogenous protein
20 in a mammalian cell.
2. A construct according to claim 1 optionally further comprising additional SV40 protein or proteins, preferably SV40 agnoprotein.

3. A construct according to claim 1 or claim 2 comprising a mixture of at least two semi-purified or pure SV40 capsid proteins.
4. A construct according to any one of claims 1 to 3 comprising a mixture of three semi-purified or pure SV40 capsid proteins.
- 5 5. A construct according to claim 1 to 4 wherein said SV40 capsid protein is semi-purified or pure VP1 or VP2 or VP3.
6. A construct according to any one of claims 1 to 5 wherein said constituent is exogenous circular or linear DNA encoding an exogenous protein or peptide product, or itself a therapeutic product, or encoding therapeutic RNA, or a vector comprising exogenous DNA encoding therapeutic RNA or encoding an exogenous protein or peptide product.
- 10 7. A construct according to claim 6 wherein said DNA is DNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount, or encodes a therapeutic RNA.
- 15 8. A construct according to claim 6 or claim 7 wherein said therapeutic protein or peptide product is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.
- 20 9. A construct according to any one of claims 6 to 8 comprising SV40-derived *ori* DNA sequence as a replication regulatory element and further comprising a DNA sequence encoding one or more regulatory elements sufficient for the
- 25

expression of said exogenous RNA or exogenous protein or peptide in said mammalian cell.

10. A construct according to any one of claims 1 to 5 wherein said constituent is exogenous RNA, wherein said RNA is RNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein or peptide product which is made contained in said cell in defective form, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount, said RNA having regulatory elements, including translation signal/s sufficient for the translation of said protein or peptide product in said mammalian cell, operatively linked thereto.
11. A construct according to claim 10 wherein said therapeutic protein or peptide product is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.
12. A construct according to any one of claims 1 to 5 wherein said constituent is an exogenous protein or peptide product.
13. A construct according to claim 12 wherein said protein or peptide product is, respectively, a therapeutic protein or peptide product which is not made or contained in said cell, or is a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is a therapeutic protein or peptide product which is made or contained in said cell in defective form or is a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount.
14. A construct according to any one of claims 1 to 5 wherein said constituent is antisense RNA or DNA or ribozyme RNA, or any RNA or DNA which

inhibits or prevents the expression of undesired protein/s in said mammalian cell.

15. A construct according to claim 14 wherein said antisense RNA is antisense RNA directed against the *bcr/abl* transcript.

5 16. A construct according to claim 14 wherein said antisense RNA is antisense RNA directed against a HIV transcript.

17. A construct according to any one of the preceding claims wherein said cell is a human cell selected from the group consisting of hemopoietic cells, epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells, tumor cells, 10 nerve cells and germ line cells.

18. A construct according to claim 17 wherein said hemopoietic cells are bone marrow cells, peripheral blood cells and cord blood cells, or liver cells.

19. A method for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid comprising the following steps:

15 a. bringing a semi-purified or pure SV40 capsid protein or a mixture of at least two such proteins into contact with said exogenous nucleic acid to give recombinant SV40 viruses or with a vector comprising said exogenous nucleic acid to give pseudoviruses; and

20 b. optionally subjecting the SV40 viruses or pseudoviruses formed in step (a) to digestion by nuclease to remove non-packaged DNA.

20. A method according to claim 19 wherein in step (a) at least one other SV40 protein, preferably SV40 agnoprotein, is added to the mixture of said SV40 capsid protein/s and said nucleic acid.

25 21. A method according to claim 19 or claim 20 wherein said SV40 capsid protein is semi-purified or pure SV40 VP1, VP2, or VP3.

22. A method according to any one of claims 19 to 21 wherein said exogenous nucleic acid is circular or linear DNA.
23. A method according to any one of claims 19 to 21 wherein said exogenous nucleic acid is RNA.
- 5 24. A method according to any one of claims 19 to 23 wherein said exogenous nucleic acid encodes a therapeutic protein or peptide product or itself a therapeutic product.
- 10 25. A method according to claim 22 wherein said DNA is DNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount or is DNA which encodes a therapeutic RNA.
- 15 26. A method according to claim 25 wherein said exogenous DNA encodes a therapeutic protein or peptide product which is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.
- 20 27. A method according to claim 19 or claim 20 wherein in step (a) SV40-derived *ori* DNA sequence is added and said exogenous nucleic acid optionally has DNA sequence encoding one or more regulatory elements sufficient for the expression of said exogenous protein in said mammalian cell operatively linked thereto.
- 25 28. A method according to claims 19 or claim 20 wherein said nucleic acid is exogenous RNA, wherein said RNA is RNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is

RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount and wherein said RNA has regulatory elements, including translation signal, sufficient for the translation of said protein product in said mammalian cell, operatively linked thereto.

29. A method for *in vitro* construction of recombinant SV40 viruses or pseudoviruses comprising an exogenous protein or peptide comprising the following steps:

- a. bringing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins into contact with said exogenous protein to give recombinant SV40 viruses or pseudoviruses; and
- b. optionally purifying the recombinant viruses or pseudoviruses obtained in step (a) from any non-packaged protein.

30. A method according to claim 29 wherein said exogenous protein or peptide are, respectively, a naturally occurring or recombinant protein or peptide, a chemically modified or peptide, or a synthetic protein or peptide.

31. A method according to claim 30 wherein said exogenous protein or peptide product are, respectively, a therapeutic protein or peptide product not made or contained in said cell, or are a therapeutic protein or peptide product made or contained in said cell in abnormally low amount, or are a therapeutic protein or peptide product made or contained in said cell in defective form or are a therapeutic protein or peptide product made or contained in said cell in physiologically abnormal or normal amount.

32. A method according to any one of claims 19 to 31 wherein said cell is a human cell selected from the group consisting of hemopoietic cells, muscle cells, tumor cells, nerve cells and germ line cells.
33. A method according to claim 32 wherein said hemopoietic cells are bone marrow cells, peripheral blood cells and cord blood cells, or liver cells.
34. A method for the *in vitro* construction of SV40 pseudoviruses comprising exogenous antisense RNA, or ribozyme RNA or RNA or DNA which inhibits or prevents the expression of undesired protein/s in a mammalian cell, comprising the following steps:
- a. bringing a semi-purified or pure SV40 capsid protein or a mixture of at least two such proteins into contact with said exogenous antisense RNA, or ribozyme RNA, or RNA or DNA which inhibits or prevents the expression of undesired protein/s in a mammalian cell, to give recombinant SV40 pseudoviruses; and
 - b. optionally subjecting the SV40 pseudoviruses formed in step (a) to digestion by nuclease to remove non-packaged DNA.
35. A method according to claim 32 wherein in step (a) at least one other SV40 protein, preferably SV40 agnoprotein, is added to the mixture of SV40 capsid protein/s and the exogenous nucleic acid or antisense nucleic acid.
36. A method according to claim 34 or claim 35 wherein said SV40 capsid protein is semi-purified or pure SV40 VP1, VP2, or VP3.
37. A method according to any one of claims 34 to 36 wherein said antisense RNA is antisense RNA directed against the *bcr/abl* transcript.
38. A method according to any one of claims 34 to 36 wherein said antisense RNA is antisense RNA directed against a HIV transcript.

39. A mammalian cell infected with a construct of any one of claims 1 to 18.
40. An infected human cell according to claim 39 selected from the group consisting of hemopoietic cells, muscle cells, tumor cells, nerve cells and germ line cells.
- 5 41. A mammalian cell infected with a construct obtained by the method of any one of claim 17 to 38.
42. An infected human cell according to claim 41 selected from the group consisting of hemopoietic cells, muscle cells, tumor cells, nerve cells and germ line cells.
- 10 43. A method of providing a therapeutic DNA, RNA, antisense RNA, ribozyme RNA, protein or peptide product to a patient in need of such product by administering to said patient a therapeutically effective amount of the SV40 viruses or pseudoviruses according to any one of claims 1 to 18.
- 15 44. A method of providing a therapeutic DNA, RNA, antisense RNA, ribozyme RNA, protein or peptide product to a patient in need of such product by administering to said patient a therapeutically effective amount of infected cells according to any one of claims 39 to 42.
- 20 45. Pharmaceutical compositions comprising as active ingredient a therapeutically effective amount of the SV40 viruses or pseudoviruses according to any one of claims 1 to 18.
46. Pharmaceutically compositions comprising as active ingredient a therapeutically effective amount of infected cells according to any one of claims 39 to claim 42.

Figure 1a



Figure 1b

2/6

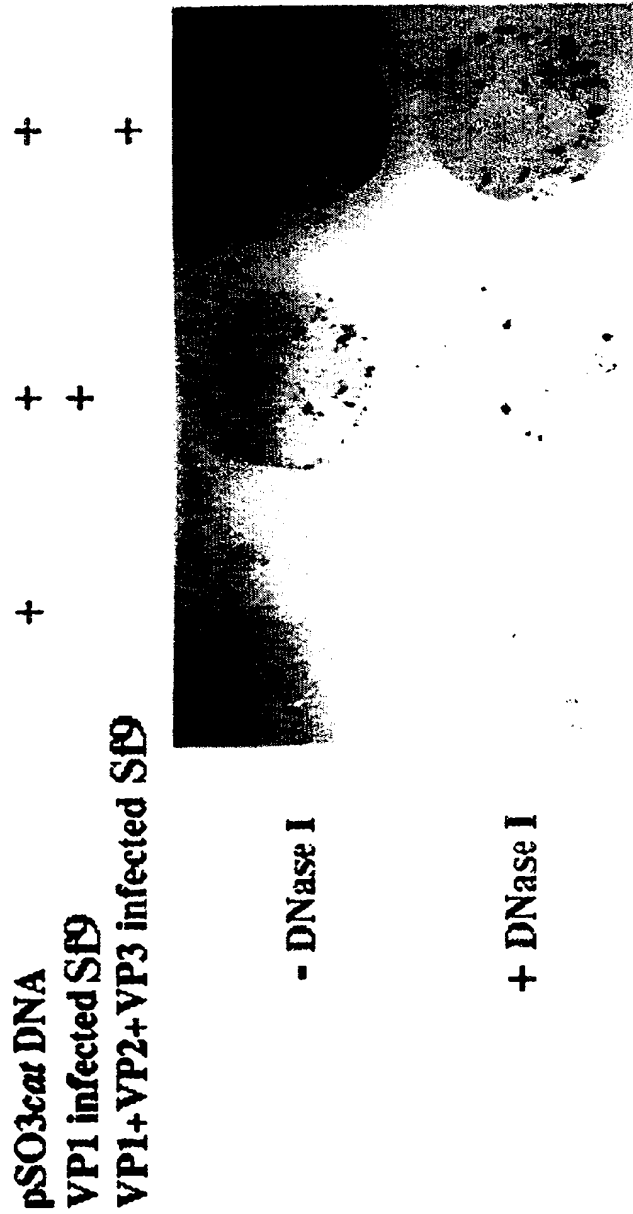


Figure 2a

3/6

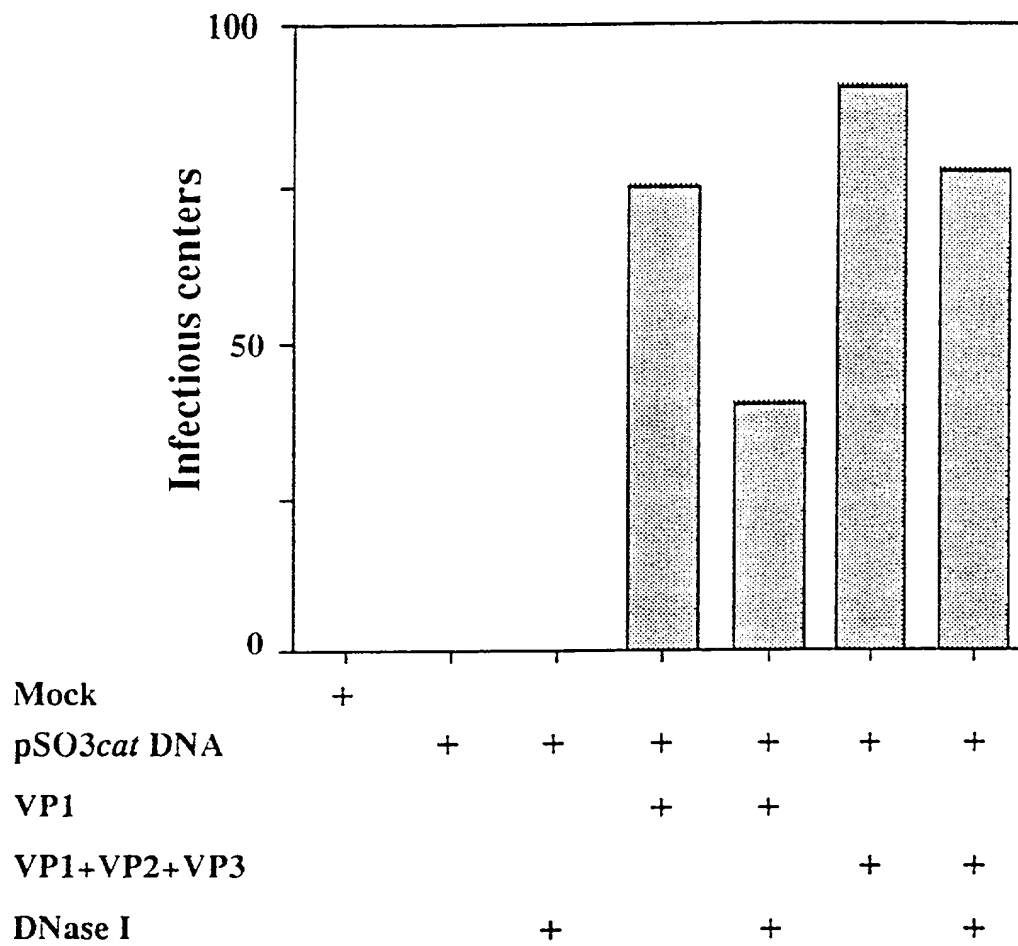


Figure 2b

4/6

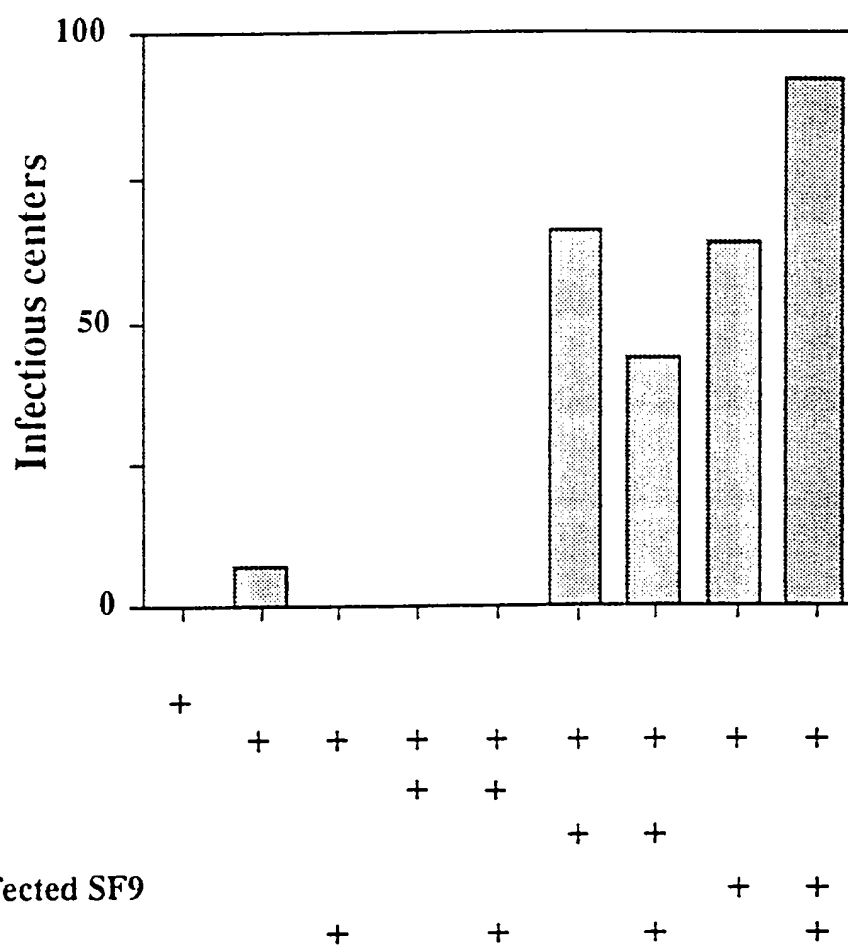
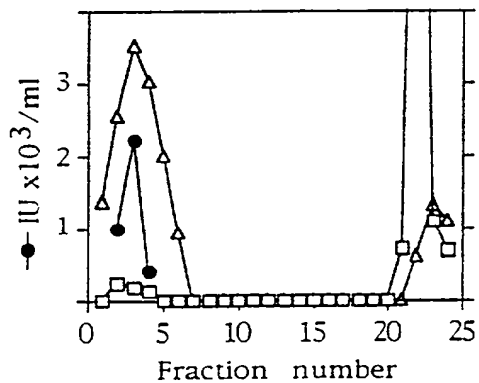
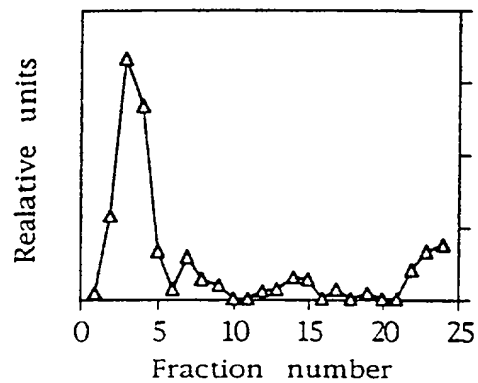
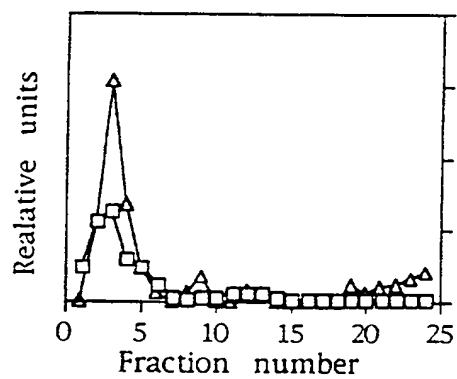
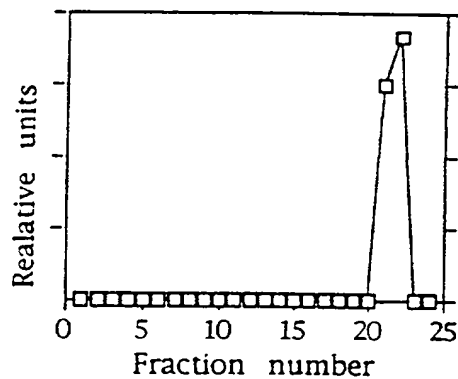


Figure 2c

5/6

**Figure 3a****Figure 3b****Figure 3c****Figure 3d**

6/6

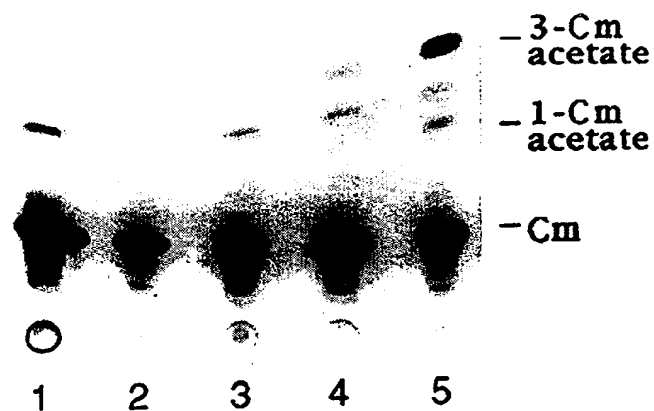


Figure 4a

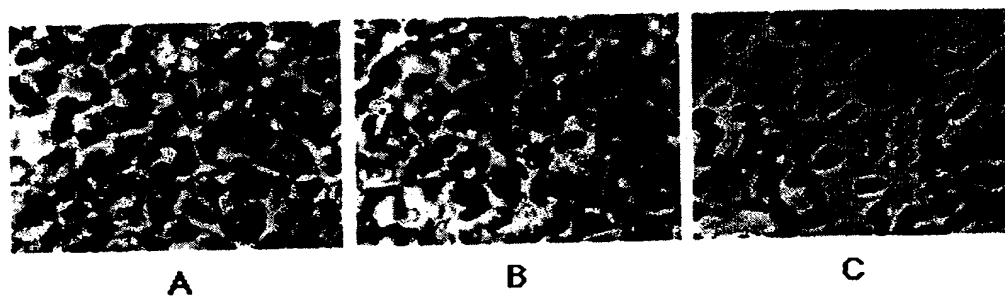


Figure 4b

DOCKET NO.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

In Vitro CONSTRUCTION OF SV40 VIRUSES AND PSEUDOVIRUSES

the specification of which (check one)

☐ is attached hereto.

☒ was filed on
which is the U.S. National Phase of International Application No.
PCT/IL96/00143 and was amended by Preliminary Amendment
on

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

<u>Prior Foreign Application(s)</u>			Priority Not Claimed
115880	Israel	6 November 1995	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year filed)	
_____	_____	_____	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year filed)	
_____	_____	_____	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year filed)	

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

_____	_____
(Application Number)	(Filing Date)
_____	_____
(Application Number)	(Filing Date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information known by me to be material to patentability as defined in 37 C.F.R. §1.56 which occurred between

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the filing date of the prior application and the national or PCT international filing date of this application:

<u>(Application Serial No.)</u>	<u>(Filing date)</u>	<u>(Status, patented, pending, abandoned)</u>
60-789,000	12-1-60	Patented
60-789,001	12-1-60	Pending
60-789,002	12-1-60	Abandoned

<u>(Application Serial No.)</u>	<u>(Filing date)</u>	<u>(Status, patented, pending, abandoned)</u>
1	1950	patented
2	1951	pending
3	1952	abandoned
4	1953	patented
5	1954	pending
6	1955	abandoned
7	1956	patented
8	1957	pending
9	1958	abandoned
10	1959	patented
11	1960	pending
12	1961	abandoned
13	1962	patented
14	1963	pending
15	1964	abandoned
16	1965	patented
17	1966	pending
18	1967	abandoned
19	1968	patented
20	1969	pending
21	1970	abandoned
22	1971	patented
23	1972	pending
24	1973	abandoned
25	1974	patented
26	1975	pending
27	1976	abandoned
28	1977	patented
29	1978	pending
30	1979	abandoned
31	1980	patented
32	1981	pending
33	1982	abandoned
34	1983	patented
35	1984	pending
36	1985	abandoned
37	1986	patented
38	1987	pending
39	1988	abandoned
40	1989	patented
41	1990	pending
42	1991	abandoned
43	1992	patented
44	1993	pending
45	1994	abandoned
46	1995	patented
47	1996	pending
48	1997	abandoned
49	1998	patented
50	1999	pending
51	2000	abandoned
52	2001	patented
53	2002	pending
54	2003	abandoned
55	2004	patented
56	2005	pending
57	2006	abandoned
58	2007	patented
59	2008	pending
60	2009	abandoned
61	2010	patented
62	2011	pending
63	2012	abandoned
64	2013	patented
65	2014	pending
66	2015	abandoned
67	2016	patented
68	2017	pending
69	2018	abandoned
70	2019	patented
71	2020	pending
72	2021	abandoned
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94	2043	patented
95	2044	pending
96	2045	abandoned
97	2046	patented
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112	2061	patented
113	2062	pending
114	2063	abandoned
115	2064	patented
116	2065	pending
117	2066	abandoned
118	2067	patented
119	2068	pending
120	2069	abandoned
121	2070	patented
122	2071	pending
123	2072	abandoned

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

I also hereby grant additional Powers of Attorney to the following attorney(s) and/or agent(s) to file and prosecute an international application under the Patent Cooperation Treaty based upon the above-identified application, including a power to meet all designated office requirements for designated states:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature _____

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